0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(8):3709–3718

doi: 10.1210/en.2005-1663

Role of Insulin Receptor in the Regulation of Glucose Uptake in Neonatal Hepatocytes

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The liver plays a major role in the regulation of glucose homoeostasis. Evidence from liver-specific insulin receptor knockout mice (LIRKO) suggested that insulin's direct and indirect effects on glucose utilization by the liver both require the presence of hepatic insulin receptors (IR). To address this issue, we have generated immortalized neonatal hepatocytes bearing (HIR LoxP) or not (HIR KO) IR. The lack of IR significantly decreased basal glucose uptake in neonatal hepatocytes from 3- to 14-d-old mice, and the expression of glucose transporter 1 (GLUT1), GLUT2, and glucokinase (GK) remained unchanged throughout development. HIR KO reconstituted hepatocytes with IR_A but not with IR_B isoform and restored basal glucose uptake up to the levels observed in HIR LoxP cells. However, both IR isoforms associated with GLUT1 or GLUT2. Overexpression of IGF-I receptor (IGF-IR) increased basal glucose uptake in neonatal hepatocytes lacking or not IR. This effect was also accompanied by its association with GLUT1 or GLUT2. Exogenous expression of GLUT4 had no effect on basal glucose uptake in neonatal hepatocytes. However, HIR LoxP hepatocytes expressing exogenous GLUT4 increased glucose uptake in the presence of insulin without showing association between GLUT4 and IR. Our data clearly indicate that IR plays a direct role in the regulation of basal glucose uptake/transport by the hepatocytes, and either type A IR or IGF-IR works on glucose uptake as a GLUT1- or GLUT2-associated cotransporter. Thus, IR mediates glucose uptake through its specific association with endogenous, but not with exogenous, glucose transporters in neonatal hepatocytes. (*Endocrinology* 147: 3709–3718, 2006)

THE LIVER PLAYS a major role in the regulation of glucose homoeostasis. Thus, under fasting conditions, hepatic glucose production (HGP), which represents 90% of total glucose release, is regulated by insulin by direct and indirect mechanisms. Conversely, glucose utilization by the liver is reduced to a minimum (8% of total glucose utilization). Deregulation between glucose uptake and production by the liver is thought to contribute to the onset of type 2 diabetes (1).

The lack of insulin receptor (IR) in a liver-specific manner, as in liver-specific IR knockout (LIRKO) mice, resulted in an impaired glucose tolerance, hyperinsulinemia, and hyperglycemia related to an increased HGP. Also, LIRKO mice showed a substantial increase in gluconeogenic gene expression accompanied by a decrease in glycolytic enzymes such as glucokinase (GK) or liver pyruvate kinase. The manifest diabetic phenotype was apparent at 2 months of age. However, this phenotype was ameliorated throughout 2–6 months, with a normoglycemic metabolism being restored after 6 months. The regression of glucose intolerance was paralleled by an increase in glucose consumption by the liver, likely because of liver dysfunction (2). Euglycemic/hyperinsulinemic clamps in LIRKO mice showed that insulin did not suppress HGP as observed in control mice. These data clearly indicated a severe hepatic insulin resistance in mice lacking IR in the liver (3). However, restoration of IR in the liver of LIRKO mice did not rescue insulin's ability to reduce glucose production *in vivo* (4). These results suggest that the role of hepatic IR and its signaling in the control of HGP in mice is limited, and consequently, indirect mechanisms of insulin-mediated inhibition of HGP are dominant (5).

Under postprandial conditions, liver removes 30–40% of the glucose that enters the portal vein after an oral glucose load and will replete its glycogen stores (6). Studies have been performed to define the relationship between insulin and net hepatic glucose uptake (NHGU) in the face of a 2-fold increase in the hepatic glucose load and in the presence or absence of the portal signal (1). There was a relationship between NHGU and the rate of portal glucose delivery as indicated by the magnitude of the arterial-portal plasma glucose gradient in 42-h fasted conscious dogs. NHGU was augmented by a rise in insulin and further stimulated by the presence of a portal signal. Thus, NHGU is dependent on the amount of glucose reaching it, the insulin level within the hepatic sinusoids, and a signal generated by portal glucose delivery.

Fed LIRKO mice also showed an impaired insulin tolerance and also a postprandial hyperglycemia. Euglycemic/hyperinsulinemic clamps also revealed a significant decrease of total glucose utilization in response to insulin in fed LIRKO mice compared with their controls, an indication of an insulin-resistance feature. These studies concluded that insulin's direct and indirect effects require the presence of hepatic IR (3). Then, a question arose as to whether or not IR

First Published Online April 27, 2006

Abbreviations: ALB, Albumin; CK18, cytokeratin 18; CPS, carbamoyl phosphate synthetase; FITC, fluorescein isothiocyanate; FS, fetal serum; GK, glucokinase; GLUT, glucose transporter; HGP, hepatic glucose production; IGF-IR, IGF-I receptor; IR, insulin receptor; IRS-1, insulin receptor substrate 1; LIRKO, liver-specific insulin receptor knockout; NHGU, net hepatic glucose uptake; Py, phosphotyrosine; VIM, vimentin.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

might play a direct role in the regulation of glucose uptake/transport by the liver.

To address this important issue, we have generated immortalized neonatal hepatocytes bearing or not IR. In addition, we have reconstituted cells with isoforms A (−exon 11) and B (+exon 11) of IR because it has been demonstrated that alternative splicing of IR is developmentally regulated (7). Our data clearly indicate that IR plays a direct role as glucose cotransporter in the regulation of glucose uptake/transport by the hepatocytes, this effect being mediated by its specific association with endogenous, but not with exogenous, hepatic glucose transporters.

Materials and Methods

Materials

FS and culture media were obtained from Life Technologies, Inc. (Gaithersburg, MD). Insulin, hygromycin, puromycin, antimouse IgG-agarose, and monoclonal anti-β-actin (clone AC-15, no. A5441) antibody were from Sigma Chemical Co. (St. Louis, MO). The anti-IGF-I receptor (anti-IGF-IR) β-chain (C-20, sc-713), anti-IR β-subunit (C-19, sc-711), anti-glucose transporter 2 (anti-GLUT2) (H-67, sc-9117), and anti-caveolin-1 (sc-894) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA.). The anti-insulin receptor substrate 1 (anti-IRS-1) (06-248), anti-phosphotyrosine (anti-Py) (clone 4G10, 05-321), and anti-IRS-2 (06-506) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-GLUT4 (CBL 243) and anti-GLUT1 (CBL 242) antibodies were from Chemicon International (Temecula, CA). The anti-phospho-Akt antibody (Ser473 no. 9271) was purchased from Cell Signaling (Beverly, MA). The anti-GK antibody was a gift of J. J. Guinovart (Barcelona, Spain). For immunofluorescence, fluorescein isothiocyanate (FITC)-conjugated sheep antimouse Ig and monoclonal antivimentin (anti-VIM) (cloneV9) antibody were from Roche Molecular Biochemicals (Mannheim, Germany), polyclonal antialbumin (anti-ALB) antibody was from Nordic Immunology Laboratories (Tilburg, The Netherlands), anticytokeratin 18 (anti-CK18) monoclonal antibody (No. 61028) was from Progen Biotechnik GmbH (Heidelberg, Germany), and monoclonal anti-carbamoyl phosphate synthetase (anti-CPS) antibody was a gift of Dr. P. Martín-Sanz (Consejo Superior de Investigaciones Científicas, Madrid, Spain). Cy3-conjugated goat antirabbit Ig, [α-^{32}P]dCTP (3000 Ci/mmol) and 2-deoxy-D[1-^{3}H]glucose (11.0 Ci/mmol) were from Amersham (Aylesbury, UK).

Generation of immortalized HIR LoxP and HIR KO hepatocyte cell lines

Primary hepatocytes were obtained from livers of 3-, 7-, and 14-d-old neonatal IR LoxP mice (homozygous for a floxed allele of exon 4 of the IR) (2) and submitted to collagenase dispersion and primary culture as previously described (8). Animals were handled in accordance with approved institutional procedures. Viral Bosc-23 packaging cells were transfected at 70% confluence by calcium phosphate coprecipitation with 3 μg/6-cm dish of the puromycin-resistance retroviral vector pBabe encoding attenuated simian virus 40 large T antigen (kindly provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA). Then, primary hepatocytes were infected at 60% confluence with polybrene-supplemented (4 μg/ml) virus for 48 h and maintained in culture medium for 72 h before selection with puromycin (1 μg/ml) for 3 wk. Then, immortalized cell lines were generated and cultured for 10–15 d with arginine-free medium supplemented with 10% FS to avoid growth of nonparenchymal cells. For *in vitro* recombination of the IR, immortalized hepatocytes IR LoxP (HIR LoxP), harboring a floxed allele of exon 4 of the IR, were first cultured to 70–80% confluence. After 24 h, cells were infected with adenoviruses encoding *cre* recombinase at a titer of 10^9 plaque-forming units. After 1 h, growth medium was added for an additional 48 h. Individual colonies were selected, and deletion of IR was assessed by Western blot. In addition, these cells (HIR KO) were cloned twice and resubmitted to viral infection to assure complete IR deletion.

Plasmids and retroviral infection

Coding sequences for the individual spliced isoforms of the human IR either containing or lacking exon 11 (isoforms B and A, respectively) and coding sequence for human IGF-IR, cloned into pBABE-hygro retroviral vector, were a gift of C. R. Kahn (Joslin Diabetes Center, Boston, MA). Viral particles were obtained as described above. HIR KO hepatocytes were infected with polybrene-supplemented (4 μg/ml) virus for 48 h and then placed in selection medium containing hygromycin (200 μg/ml) for at least 2 wk.

Immunofluorescence and confocal imaging

Cells were grown in glass coverslips until 80% confluence was reached. Then, cells were washed twice with PBS, fixed in methanol (−20 C) for 2 min and processed to immunofluorescence. Primary antibodies (anti-ALB, anti-CPS, anti-CK18, and anti-VIM) were applied for 1 h at 37 C in PBS/1% BSA, followed by four 5-min washes in PBS, a 45-min incubation with fluorescence-conjugated antibodies (FITC-conjugated sheep antimouse and Cy3-conjugated goat antirabbit), and four final washes of 5 min each in PBS. Immunofluorescence was examined in an MRC-1024 (Bio-Rad, Hempstead, UK) confocal microscope adapted to an inverted Nikon Eclipse TE 300 microscope. Images were taken with 488-nm laser excitation for FITC-conjugated antibodies and 514-nm laser excitation for Cy3-conjugated antibodies. Fluorescence emissions were detected through a 513/24-nm bandpass filter for FITC and a 605/15-nm bandpass filter for Cy3.

Immunoprecipitations and Western blot

Quiescent cells (20 h serum-starved) were treated without or with several doses of insulin and lysed as previously described (9). After protein content determination, equal amounts of protein (600 μg to 1 mg) were immunoprecipitated at 4 C with the corresponding antibodies. The immune complexes were collected on protein A-agarose or antimouse IgG-agarose beads and submitted to SDS-PAGE. Proteins were transferred to Immobilon membranes and incubated overnight with several antibodies as indicated. Immunoreactive bands were visualized using the ECL Western blotting protocol (Amersham).

Protein determination

Protein determination was performed by the Bradford dye method (10), using the Bio-Rad reagent and BSA as the standard.

Transduction of HIR LoxP and HIR KO hepatocytes by adenoviral infection

Neonatal hepatocytes were infected with adenoviruses encoding GLUT4 (a generous gift of A. M. Gomez-Foix, University of Barcelona, Barcelona, Spain) or mock (adenovirus encoding β-galactosidase) at a titer of 10^9 plaque-forming units. Cells (80–90% confluence) were routinely infected for 24–48 h as previously described (11).

RNA extraction and Northern blot analysis

At the end of the culture time, total RNA was isolated as described (12) and submitted to Northern blot analysis. Blots were hybridized with a probe for GLUT4 or 18S rRNA as a loading control.

Semiquantitative RT-PCR

To analyze endogenous (mouse) and reconstituted (human) IR isoforms in immortalized hepatocytes by PCR, 5 μg total RNA was primed with oligodeoxythymidine in the presence of murine mammary tumor virus reverse transcriptase (Invitrogen, Carlsbad, CA) to synthesize cDNA. The samples were diluted 5-fold, and 5% of the total volume was used for subsequent PCR. Primers used were mouse IR exon 11 primer 1, 5′-ATCAGAGTGAGTATGACGACTCGG-3′, and primer 2, 5′-TCCTGACTTGTGGGCACAATGGTA-3′; and human IR exon 11 primer 1, 5′-ACCAGAGTGAGTATGAGGATTCGG-3′, and primer 2, 5′-TCCGGACTCGTGGGCACGCTGGTC-3′. PCR were performed as described (13). Reaction products were resolved on 2% agarose gels.

Subcellular fractionation

Cells were washed with ice-cold PBS and scraped in homogenization buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After 10 min incubation, cells were homogenized with 30 strokes of a Dounce homogenizer using a tight-fitting pestle. Nuclei were collected by centrifugation at 500 × *g* for 5 min, and the low-speed supernatant was centrifuged at 100,000 × *g* for 30 min. The high-speed supernatant constituted the cytosolic fraction. The pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 60 min. The Triton-soluble component (plasma membrane fraction) was separated from the Triton-insoluble material (cytoskeletal fraction) by centrifugation at 100,000 × *g* for 15 min. Plasma membrane and cytosolic fractions were kept at −70 C before protein quantification and Western blotting.

Measurement of glucose transport or uptake

Cells were cultured for 20 h in serum-free DMEM (5 mM glucose) and then treated or not with 10 nM insulin or 10 nM IGF-I for 2 (glucose transport) or 10 (glucose uptake) minutes. Glucose transport/uptake was measured by incubating cells with 2-deoxy-d-[1-^{3}H]glucose for 2 or 10 min, respectively, in triplicate dishes from four to six independent experiments as previously described (14).

Statistical analysis

Statistically significant differences between mean values were determined using paired Student's *t* test.

Results

Generation and characterization of HIR LoxP and HIR KO immortalized hepatocyte cell lines

Immortalized neonatal hepatocyte cell lines (HIR LoxP) have been generated from livers of IR loxP mice at 3, 7, and 14 d after birth. All cell lines were obtained after infection of neonatal hepatocytes in primary culture with retrovirus encoding attenuated large T antigen, followed by antibiotic (puromycin) selection for at least 3 wk. Then, immortalized cells were cultured for 10–15 d in arginine-free medium supplemented with epidermal growth factor (20 ng/ml) to select hepatocytes having a functional urea cycle. Next, we deleted IR by infection with an adenovirus encoding Cre recombinase, generating HIR KO cell lines. First, we proceeded to characterize the phenotype of all these cell lines. A photograph of growing cells, at different stages of development, is shown in Fig. 1A. Then, we performed immunofluorescence of growing cells with a set of antibodies against metabolic and cytoskeletal markers. Figure 1B shows the phenotypical characterization of 3-d-old immortalized HIR LoxP and HIR KO neonatal hepatocytes. HIR LoxP and HIR KO neonatal hepatocytes express ALB (a plasma protein secreted exclusively by hepatocytes) as well as CPS (a urea

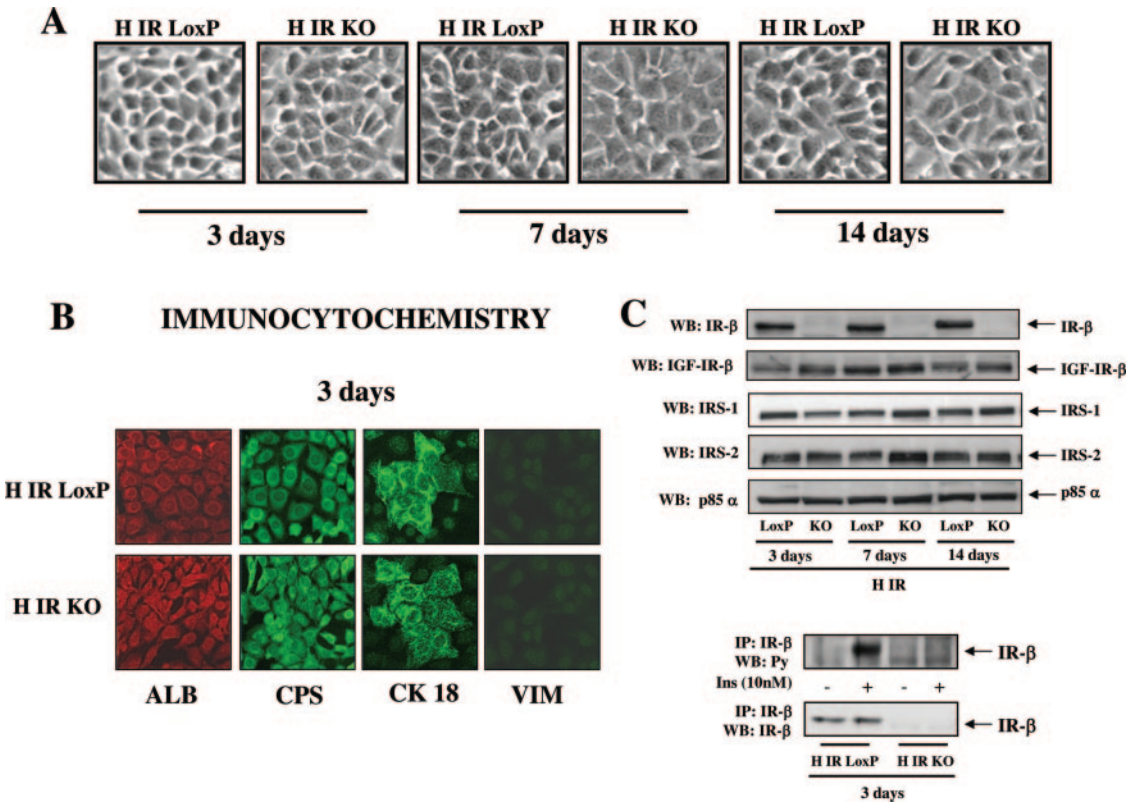

FIG. 1. Characterization of immortalized hepatocyte cell lines. A, Photograph of growing HIR LoxP and HIR KO neonatal hepatocytes from 3-, 7-, and 14-d-old mice; B, immunofluorescence detection of ALB, CPS, CK18, and VIM of growing HIR LoxP and HIR KO neonatal hepatocytes from 3-d-old mice; C, *top*, cells were grown to confluence in the presence of 10% FS, and whole-cell lysates (100 μg of protein) were submitted to SDS-PAGE and analyzed by Western blot (WB) using antibodies against IR β-chain, IGF-IR β-chain, IRS-1, IRS-2, and p85α; *bottom*, quiescent HIR LoxP and HIR KO neonatal hepatocytes from 3-d-old mice were stimulated with 10 nM insulin for 5 min. Control cells were cultured in the absence of the hormone. At the end of the culture time, cells were lysed and 1 mg total protein was immunoprecipitated (IP) with anti-IR β-chain antibody. The resulting immune complexes were analyzed by Western blotting with anti-Py or anti-IR-β antibodies. All the results shown are representative of three experiments.

cycle marker), indicating that after the immortalization these cells maintain hepatocyte phenotypic features. Moreover, all cell lines maintained the expression of the cytoskeletal marker CK18, indicating the presence of epithelial parenchymal cells (15). Conversely, all cell lines showed negative immunofluorescence with the anti-VIM antibody. VIM is a cytoskeletal marker characteristic of cells from mesenchymal origin and should be absent in parenchymal hepatocytes, to confirm that the maintenance of cell lines in arginine-free medium has been sufficient to eliminate a possible contamination of fibroblasts in the primary culture.

The deletion of IR was confirmed by Western blot analysis. As depicted in Fig. 1C, the lack of IR β-chain expression was detected in HIR KO neonatal hepatocytes; meanwhile, IGF-IR expression remained unchanged in all cell lines at different stages of development. Also, the postreceptor downstream insulin signaling machinery, as represented by IRS-1, IRS-2, and p85α, remained unchanged in neonatal hepatocytes throughout development. Moreover, in HIR KO cells from 3-d-old mice, no IR β-chain tyrosine phosphorylation was found after stimulation with 10 nM insulin. Hepatocytes from 7- and 14-d-old mice showed a similar pattern of cellular and insulin signaling markers (results not shown).

Effect of deletion of IR on glucose transport or uptake in neonatal hepatocytes

Hepatic glucose uptake and utilization is essential to maintain whole-body glucose homeostasis. Accordingly, we next investigated whether the lack of IR could unbalance the rate of glucose entry in neonatal hepatocytes. For that goal, HIR LoxP and HIR KO cells were cultured up to 80% confluence and then glucose uptake was measured after 20 h of culture in 5 mM glucose DMEM. The rationale for using 5 mm glucose was to simulate its physiological relevance at the portal vein from suckling animals. As shown in Fig. 2A, the lack of IR decreased by 50% basal glucose uptake in neonatal hepatocytes from 3-d-old mice. Also, a 30% decrease in cells from 7- and 14-d-old mice was observed, as compared with their corresponding HIR LoxP controls. Interestingly, basal glucose uptake decreased significantly in HIR LoxP neonatal hepatocytes from 7- and 14-d-old mice, compared with HIR LoxP cells from 3-d-old mice. Although IR mRNA was still present in KO cells, because deletion of exon 4 by cre recombination creates a frameshift mutation predicting a premature stop codon, the ratio of IR_A to IR_B isoforms did not change among the several HIR LoxP mouse hepatocyte cell lines from different stages of development, as revealed by semiquantitative PCR (Fig. 2A, *right inset*). Importantly, HIR LoxP neonatal hepatocytes showed a similar profile of IR_A and IR_B isoforms to that of wild-type immortalized neonatal hepatocytes. As expected, glucose uptake in neonatal hepatocytes was insulin independent (Fig. 2B). To investigate whether the lack of IR in hepatocytes could differentially affect glucose transport and/or glucose uptake, HIR LoxP and HIR KO cells were incubated with [^{3}H]2-deoxyglucose for 2 or 10 min, respectively. The results depicted in Fig. 2C indicated that the lack of IR equally down-regulates glucose uptake (10 min) or transport (2 min) in hepatocytes. Moreover, the ubiquitously expressed GLUT1, the liver-specific GLUT2, and GK expression remained unchanged in all cell lines bearing or not IR. In addition, the expression of glucose 6-phosphatase was very modest in neonatal hepatocytes under the experimental conditions herein described (results not shown). All these data reinforce the notion that glucose uptake is much affected by the lack of IR in neonatal hepatocytes but not by a dysregulation in glucose phosphorylation/dephosphorylation.

Differential restoration of basal glucose uptake by reconstitution with IR_A and IR_B isoforms in HIR KO neonatal hepatocytes

To determine whether or not IR splice isoforms have a unique role in glucose uptake in hepatocytes, HIR KO cells were infected with retroviruses encoding for human IR_A or IR_B. A representative Western blot of the levels of IR expression in reconstituted cells is shown (Fig. 3A, *left*). Moreover, specific reconstitution with IR_A or IR_B isoforms was assessed by PCR (Fig. 3A, *right*). To assess the functionality of reconstituted insulin receptors, we analyzed the response of reconstituted hepatocyte cell lines to insulin in inducing tyrosine phosphorylation of IR. Quiescent cells were stimulated with 10 nM insulin for 5 min and then lysed. Equal amounts of protein (1 mg) were immunoprecipitated with the anti-IR β-chain antibody, and tyrosine phosphorylation of IR was analyzed by anti-Py Western blot. As shown in Fig. 3B (*left*), insulin was able to stimulate tyrosine phosphorylation of IR in HIR KO reconstituted hepatocytes with both splice isoforms of IR (IR_A and IR_B) to the same extent as in HIR LoxP hepatocytes. As expected, no effect of insulin was observed on tyrosine phosphorylation of IR in HIR KO hepatocytes. In addition, reconstitution of HIR KO hepatocytes with either IR_A or IR_B rescued Akt phosphorylation in response to insulin. These data clearly indicate that both isoforms of IR were functional in reconstituted neonatal hepatocytes.

The glucose transporter GLUT2 is a transmembrane protein that facilitates the net hepatic efflux of glucose. The coimmunoprecipitation of IR and GLUT2 in rat hepatocytes has recently been demonstrated (16). Here, we studied whether this association occurs in HIR LoxP or HIR KO reconstituted hepatocytes with IR_A or IR_B isoforms, respectively. As a negative control, we used HIR KO hepatocytes. Cell lysates were obtained from the corresponding hepatocyte cell lines, and 1 mg total protein was immunoprecipitated with anti-GLUT2 antibody. The resulting immune complexes were analyzed by Western blot with the anti-IR β-chain antibody. The coimmunoprecipitation IR/GLUT2 band was observed in HIR LoxP and HIR KO reconstituted hepatocytes with IR_A and IR_B isoforms, respectively, but not in HIR KO cells (Fig. 3B, *right*). To analyze whether this coimmunoprecipitation could also involve the ubiquitously expressed GLUT1, we performed the same experiment by immunoprecipitating whole-cell lysates with the anti-GLUT1 antibody. As depicted in Fig. 3B (*right*), we also observed GLUT1/IR_A and GLUT1/IR_B immune complexes in neonatal hepatocytes.

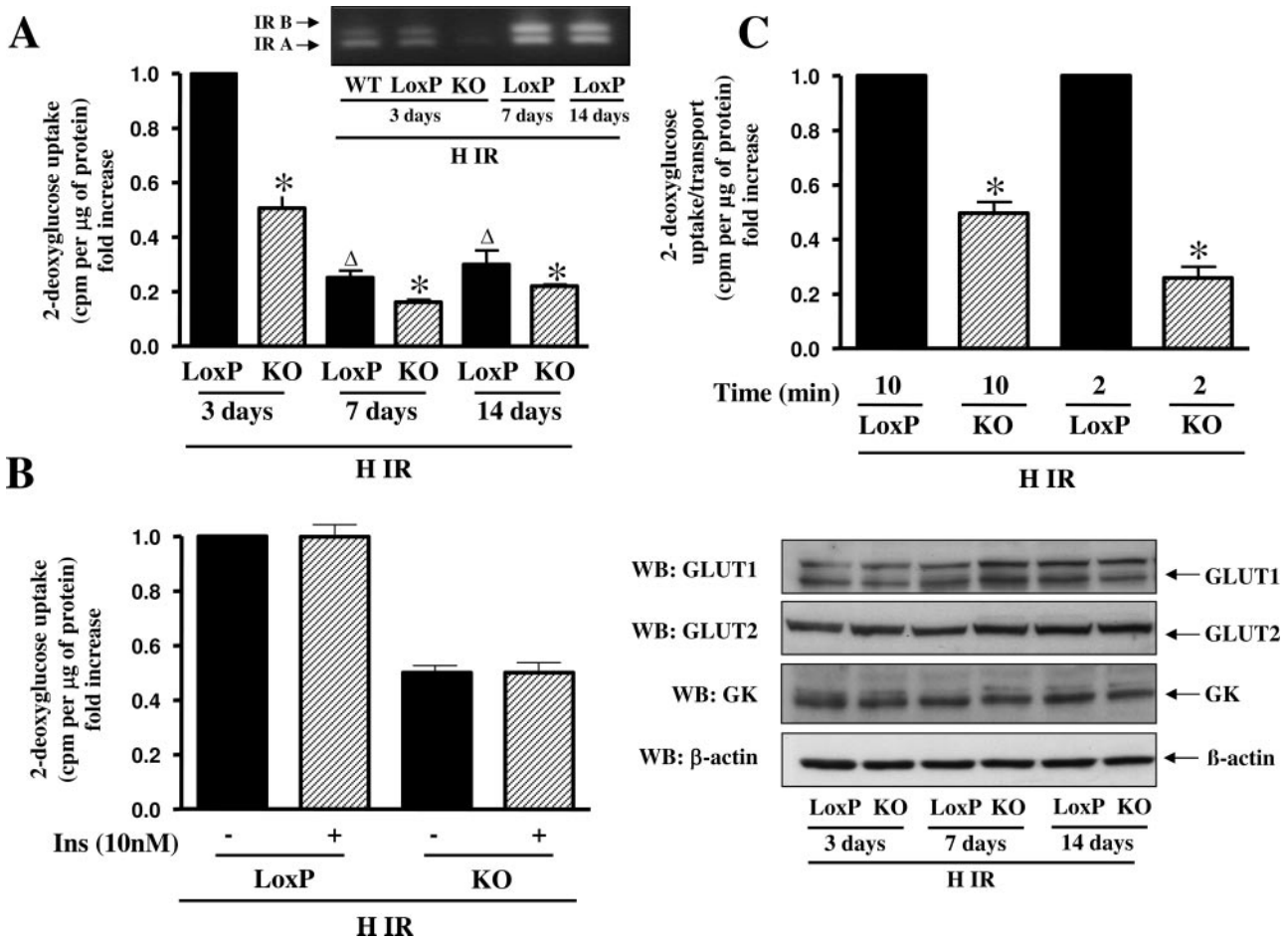

FIG. 2. Effect of IR deletion on basal glucose uptake or transport in neonatal hepatocytes. A, *Left*, Immortalized HIR LoxP neonatal hepatocytes obtained from 3-, 7-, and 14-d-old mice were cultured to 80% confluence and then serum-starved in 5 mM DMEM for 15 h. Glucose uptake was measured as described in *Materials and Methods*. Results are expressed as cpm/μg total protein and are means ± SE from three independent experiments with duplicate dishes. Statistical significance was carried out by Student's *t* test by comparison of HIR KO with HIR LoxP cells from 3-, 7-, and 14-d-old mice, respectively (*, $P < 0.05$) or HIR LoxP cells from 7- and 14-d-old mice *vs.* HIR LoxP cells from 3-d-old mice (Δ, $P < 0.05$). *Inset*, Total RNA was isolated from HIR LoxP (from 3-, 7-, and 14-d-old mice), HIR KO (from 3-d-old mice), and wild-type (WT) (from 3-d-old mice) neonatal hepatocytes, and RT-PCR was performed as described in *Materials and Methods*. A representative experiment is shown. B, Hepatocytes were serum starved for 12–15 h in 5 mM glucose DMEM and stimulated with 10 nm insulin (Ins). Glucose uptake was measured, and results are expressed as cpm/μg total protein and are means ± SE from three independent experiments with duplicate dishes. C, *Top*, HIR LoxP and HIR KO cells were incubated with a mix of [^{3}H]glucose and cold glucose for 2 or 10 min. Glucose transport/uptake was measured, and results are expressed as cpm/μg total protein and are means ± SE from three independent experiments with duplicate dishes. Statistical significance was carried out by Student's *t* test by comparison of HIR KO with HIR LoxP cells (*, $P < 0.05$). *Bottom*, Cells were grown to confluence in the presence of 10% FS. Whole-cell lysates (100 μg protein) were submitted to SDS-PAGE and analyzed by Western blot (WB) using antibodies against GLUT1, GLUT2, GK, and β-actin. A representative experiment of three is shown.

Our next goal was to investigate whether there were differences in the subcellular localization of IR isoforms with glucose transporters. Accordingly, we performed a subcellular fractionation protocol to separate plasma membrane and cytosolic fractions from HIR KO hepatocytes reconstituted with IR_A or IR_B isoforms as well as from HIR LoxP and HIR KO cells as controls. Then, equals amount of protein from each fraction were immunoprecipitated with anti-GLUT2 antibody and subsequently analyzed by Western blot with the anti-IR β-chain antibody. Figure 3B (*right*) reveals that no differences were observed in the association IR_A or IR_B isoforms with GLUT2, with the protein band being visualized in the plasma membrane fraction but not in the cytosol.

To assess that IR plays a direct role in glucose uptake, we investigated whether reconstituted HIR KO hepatocytes with IR_A and IR_B isoforms were able to restore basal glucose uptake. As depicted in Fig. 3C, HIR KO reconstituted hepatocytes with IR_A isoform restored basal glucose uptake up to the levels observed in HIR LoxP cells. However, no effect was observed in reconstituted hepatocytes with the IR_B isoform.

Overexpression of IGF-IR increases basal glucose uptake in HIR LoxP and HIR KO hepatocytes

IR and IGF-IR are highly homologous tyrosine kinase receptors and activate many of the same signaling cascades in target cells. In fact, IGF-I activates glucose uptake in insulin-sensitive cells such as brown adipocytes (17). Accordingly,

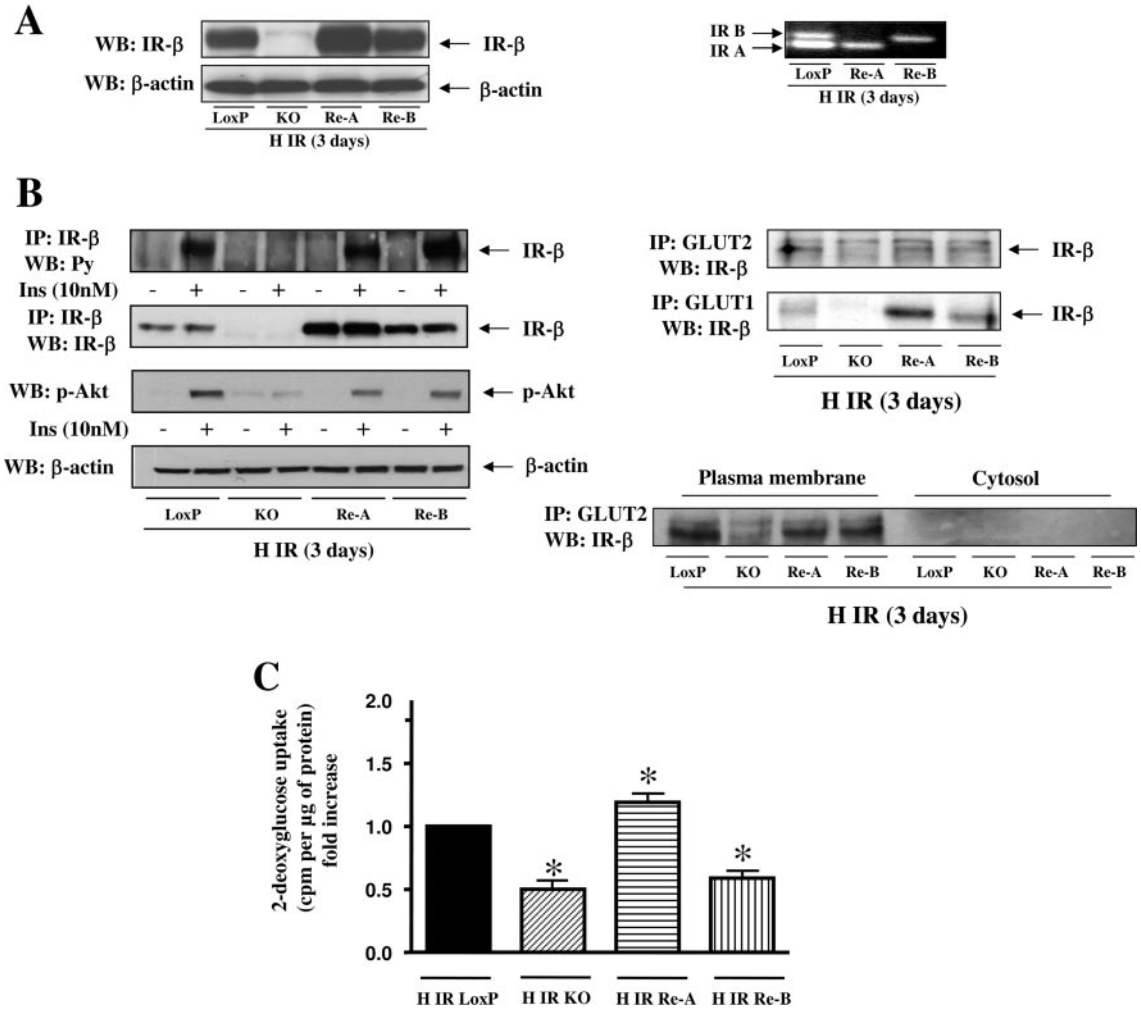

FIG. 3. Differential restoration of basal glucose uptake by reconstitution of IR_A and IR_B isoforms in HIR KO neonatal hepatocytes. A, *Left*, HIR KO hepatocytes were infected with a retroviral vector encoding IR_A or IR_B isoforms. Hygromycin-resistant clones were expanded, and cell lysates were analyzed by Western blot (WB) with anti-IR β-chain antibody. *Right*, Reconstitution of HIR KO hepatocytes with IR_A or IR_B isoforms was analyzed by RT-PCR as described in *Materials and Methods*. B, *Left*, Quiescent (20-h serum-starved) hepatocytes were stimulated with insulin (10 nM) for 5 min. Control cells were cultured in the absence of the hormone. At the end of the culture time, cells were lysed, and 1 mg total protein was immunoprecipitated (IP) with anti-IR-β chain antibody. The resulting immune complexes were analyzed by Western blotting (WB) with anti-Py or anti-IR β-chain antibodies, as indicated in each panel. Phosphorylation of Akt was measured by Western blot with the anti-phospho-Akt (ser473) antibody. The results shown are representative of three experiments. *Right*, HIR LoxP, HIR KO, and reconstituted cells HIR KO Re-A and HIR KO Re-B neonatal hepatocytes were lysed, and 1 mg of total protein was immunoprecipitated with anti-GLUT2 or anti-GLUT1 antibodies. The resulting immune complexes were analyzed by Western blotting with anti-IR β-chain antibody, as indicated in each panel. A representative experiment of three is shown. Cells were collected for subcellular fractionation. Equals amount of protein from plasma membrane and cytosolic fractions were immunoprecipitated with the anti-GLUT2 antibody and analyzed by Western blot with the anti-IR β-chain antibody. The experiment was repeated three times with similar results. C, HIR LoxP, HIR KO, and reconstituted cells (HIR KO Re-A and HIR KO Re-B) were serum starved for 12–15 h in 5 mM glucose DMEM. Glucose uptake was measured, and results are expressed as cpm/μg total protein and are means ± SE from three independent experiments with duplicate dishes. Statistical significance was carried out by Student's *t* test by comparison of HIR KO Re-A and HIR KO Re-B cells *vs.* HIR KO. *, $P < 0.05$.

our next purpose was to investigate whether overexpression of IGF-IR could compensate for the decrease in basal glucose uptake observed in HIR KO neonatal hepatocytes lacking IR. Thus, we overexpressed IGF-IR in both HIR LoxP and HIR KO cells by means of retroviral infection, generating HIR LoxP (IGF-IR) and HIR KO (IGF-IR) hepatocyte cell lines, respectively. Neonatal hepatocytes lacking or not IR expressed similar levels of IGF-IR. After retroviral infection, IGF-IR expression was increased by 2-fold in both cell lines (Fig. 4A, *top*). Then, we performed anti-IGF-IR Western blot from anti-GLUT2 or anti-GLUT1 immunoprecipitates. As shown in Fig. 4A (*bottom*), IGF-IR coimmunoprecipitated with GLUT2 or GLUT1 in neonatal hepatocytes. These results indicate that besides IR/GLUT2 or IR/GLUT1 complex formation, IGF-IR can also associate with either GLUT1 or GLUT2 independently of IR. Then, we measured basal glucose uptake in HIR LoxP (IGF-IR) and HIR KO (IGF-IR) cells. As shown in Fig. 4B, overexpression of IGF-IR increased basal glucose uptake in neonatal hepatocytes regardless of IR.

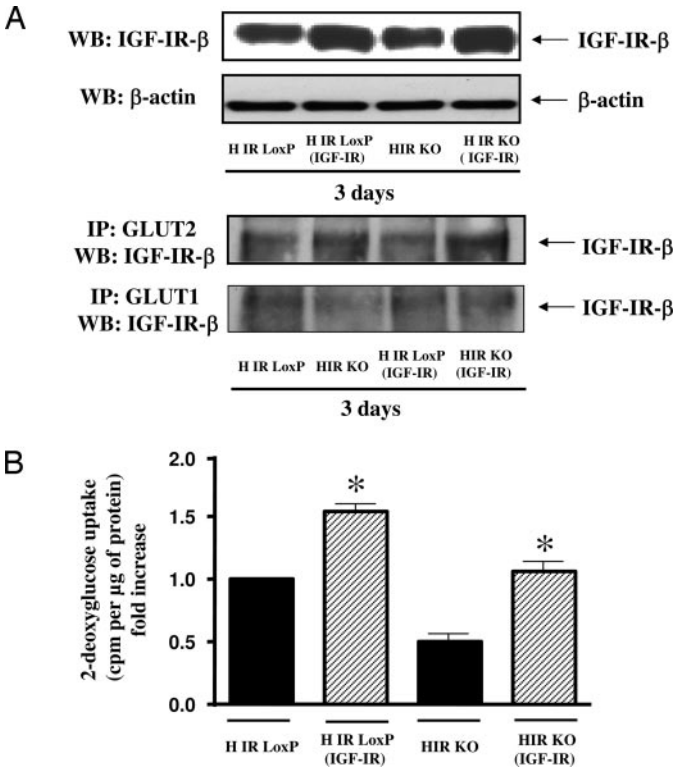

FIG. 4. Overexpression of IGF-IR increases basal glucose uptake in HIR LoxP and HIR KO neonatal hepatocytes. A, *Top*, HIR KO hepatocytes were infected with a retroviral vector encoding IGF-IR. Hygromycin-resistant clones were expanded, and cell lysates were analyzed by Western blot (WB) with anti-IGF-IR β-chain antibody. *Bottom*, Cells were lysed, and 1 mg total protein was immunoprecipitated (IP) with anti-GLUT1 or anti-GLUT2 antibodies. The resulting immune complexes were analyzed by Western blotting with anti-IGF-IR β-chain antibody, as indicated in each panel. The results shown are representative of three experiments. B, HIR LoxP, HIR LoxP (IGF-IR), HIR KO, and HIR KO (IGF-IR) hepatocytes were serum starved for 12–15 h in 5 mM glucose DMEM. Glucose uptake was measured, and results are expressed as cpm/μg total protein and are means ± SE from three independent experiments with duplicate dishes. Statistical significance was carried out by Student's *t* test by comparison of HIR LoxP (IGF-IR) and HIR KO (IGF-IR) cells *vs.* HIR LoxP and HIR KO, respectively. *, $P < 0.05$.

Exogenous expression of GLUT4 drives insulin and IGF-I-induced glucose uptake in HIR LoxP and HIR KO, respectively

Insulin stimulates glucose uptake in muscle and adipose tissues through translocation of GLUT4 from an intracellular pool into the plasma membrane (18). Because liver lacks GLUT4, our next aim was to investigate whether or not IR could associate with exogenously expressed GLUT4. For that goal, HIR LoxP and HIR KO hepatocytes were infected with adenoviruses encoding GLUT4 or empty vector (mock). As shown in Fig. 5A, both cell lines transduced with GLUT4 adenoviruses expressed GLUT4 mRNA, as revealed by a representative Northern blot analysis. More importantly, GLUT4 protein expression was detected in HIR LoxP and HIR KO neonatal hepatocytes transduced with GLUT4 adenoviruses but not with mock adenoviruses. Then, we studied whether the association of IR/GLUT4 or IGF-IR/GLUT4 occurs in HIR LoxP or HIR KO, respectively, under basal conditions or upon insulin/IGF-I stimulation. Neonatal hepatocytes were transduced with GLUT4 or mock adenoviruses for 48 h. Then, HIR LoxP and HIR KO cells were stimulated with 10 nM insulin or 10 nm IGF-I, respectively. Whole-cell lysates were immunoprecipitated with anti-GLUT4 antibody, and the resulting immune complexes were analyzed by Western blot with the anti-IR β-chain (HIR LoxP cells) or anti-IGF-IR β-chain (HIR KO cells) antibodies. As shown in Fig. 5A (*bottom*), IR/GLUT4 or IGF-IR/GLUT4 coimmunoprecipitates were not observed regardless of insulin or IGF-I stimulation in the corresponding cells.

To assess whether the lack of association between exogenously expressed GLUT4 and IR or GLUT4 and IGF-IR may be accounted for by a distinctive cellular compartmentation, we studied GLUT4 protein subcellular localization in transduced hepatocytes under basal conditions and upon insulin or IGF-I stimulation. Cells were serum starved for 12–15 h and stimulated with 10 nM insulin (HIR LoxP cells) or 10 nm IGF-I (HIR KO cells) for 15 min, respectively. Then, we performed a subcellular fractionation protocol to separate plasma membrane and cytosolic fractions of GLUT4-positive hepatocytes. As shown in Fig. 5B, a GLUT4 protein band was visualized only in the plasma membrane fraction in either nonstimulated or stimulated cells. Finally, we investigated whether GLUT4-expressing hepatocytes were able to increase glucose uptake in response to insulin or IGF-I. For that goal, HIR LoxP and HIR KO neonatal hepatocytes were transduced with adenoviruses encoding GLUT4 or mock adenoviruses. Forty-eight hours later, cells were serum starved in 5 mM glucose DMEM for 12–15 h and stimulated for 20 min with 10 nm insulin (HIR LoxP) or 10 nM IGF-I (HIR KO), respectively. As depicted in Fig. 5C, transduction with GLUT4 adenovirus did not affect basal glucose uptake in both cell types. However, insulin and IGF-I treatment significantly increased glucose uptake in HIR LoxP and HIR KO, respectively.

Discussion

The liver plays a major role in insulin-regulated glucose homoeostasis through the balance between glucose utilization and glucose production, both processes being tightly coordinated. However, whereas the direct and/or indirect effects of insulin on HGP are widely recognized, uncertainty remains regarding its direct or indirect role in glucose disposal by the liver (1).

Euglycemic/hyperinsulinemic clamp studies in LIRKO mice showed a severe insulin resistance either in total glucose utilization or HGP. Thus, the lack of IR and its signaling break down the balance between glucose utilization and glucose production by the liver. The final outcome was a diabetic phenotype showing a severe compensatory hyperinsulinemia, accompanied by postprandial and fasting hyperglycemia (2). These data strongly suggest that IR by itself is involved in both glucose disposal and glucose output by the liver. On the other hand, liver-specific phosphoinositide-dependent protein kinase 1 knockout mice just recently showed an impaired glucose tolerance, but insulin tolerance. Thus, the defect in insulin signaling impaired the inhibition of gluconeogenic enzymes and glucose output in response to

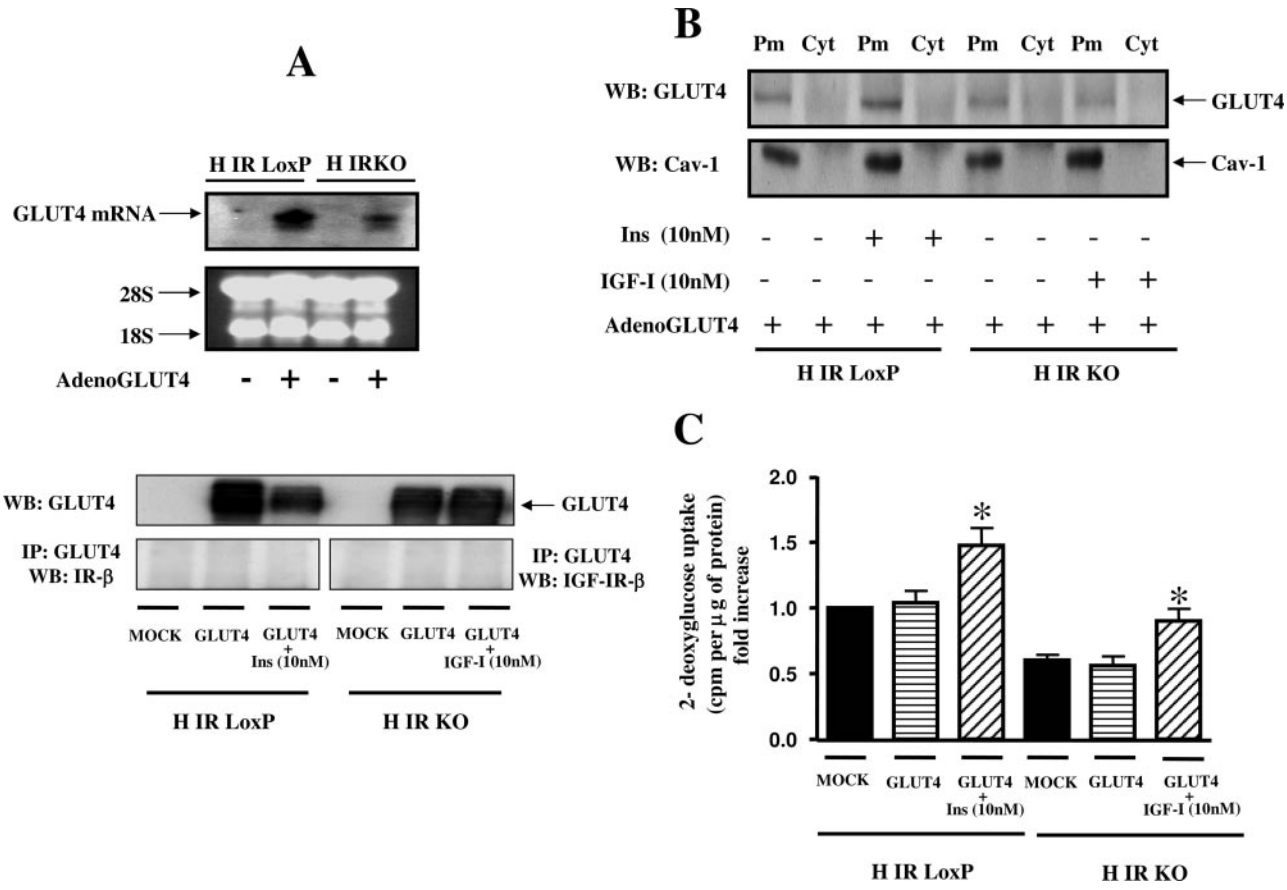

FIG. 5. Exogenous expression of GLUT4 drives an insulin- and IGF-I-induced glucose uptake in HIR LoxP and HIR KO neonatal hepatocytes, respectively. A, *Top*, HIR LoxP and HIR KO neonatal hepatocytes were infected with adenoviruses encoding mock and GLUT4, and 48 h after infection, GLUT4 mRNA level was analyzed by Northern blot. A representative experiment is shown. *Bottom*, At 48 h after transduction, cells were stimulated with 10 nM insulin (HIR LoxP) or 10 nm IGF-I (HIR KO) for 15 min. Cells were lysed, and GLUT4 protein content was analyzed by Western blot (WB). Total protein (1 mg) was immunoprecipitated (IP) with anti-GLUT4 antibody. The resulting immune complexes were analyzed by Western blotting with anti-IR-β (HIP LoxP) or anti-IGF-IR-β (HIR KO) antibodies, as indicated in each panel. The results shown are representative of three experiments. B, At 48 h after transduction, cells were stimulated with 10 nM insulin (HIR LoxP) or 10 nm IGF-I (HIR KO) for 15 min and collected for subcellular fractionation. Ten micrograms of plasma membrane and cytosolic proteins of each condition were subjected to SDS-PAGE and analyzed by Western blot with the anti-GLUT4 and anti-caveolin-1 antibodies. A representative experiment is shown. C, Cells were cultured as described in B. Glucose uptake was measured, and results are expressed as cpm/μg total protein and are means $\pm$ SE from three independent experiments with duplicate dishes. Statistical significance was carried out by Student's *t* test by comparison of insulin-stimulated HIR LoxP (GLUT4) and IGF-I-stimulated HIR KO (GLUT4) cells *vs.* nonstimulated cells. *, $P < 0.05$.

insulin by the liver without insulin resistance. Accordingly, these mice show normal insulinemia or postprandial/fasting glycemia (19). Taken together, these results seem to suggest that IR rather than insulin signaling regulates glucose disposal by the liver. However, deletion of IR in the liver greatly affects GK protein content, whose gene expression requires insulin signaling (2). Therefore, a direct role of insulin signaling in the basal glucose transport/uptake by the liver cannot be ruled out.

Our results provide strong evidence regarding the direct role of IR in controlling glucose uptake by the hepatocytes. More importantly, the decrease in basal glucose uptake/transport in neonatal hepatocytes occurred without changes in the expression of GLUT1, GLUT2, and GK. In fact, glucose metabolism is required for regulation of GLUT2 expression in the liver *in vivo* and *in vitro* (20). In addition, neonatal hepatocytes virtually lack the expression of gluconeogenic enzymes such as glucose 6-phosphatase under the experimental conditions assayed. These results point out that glucose transport/uptake rather than glucose phosphorylation/dephosphorylation is compromised by the lack of IR in neonatal hepatocytes. On the other hand, this effect was observed in a postnatal development-dependent manner. However, the decrease in basal glucose uptake observed in 3-d-old neonatal hepatocytes lacking IR was less prominent in cells from 7- and 14-d-old mice. In fact, suckling newborns show a marked hypoinsulinemia and, consequently, very limited insulin-induced glucose consumption by extrahepatic tissues. Whether the functional impact of the IR regarding glucose uptake can be extrapolated to adult hepatocytes remains to be established.

Reconstitution of HIR KO cells with insulin receptor A isoform (Ex11−) (IR_A) restored basal glucose uptake up to the levels observed in wild-type hepatocytes. Conversely, reconstitution with insulin receptor B isoform (Ex11+) (IR_B) had no effect on glucose uptake. However, both IR isoforms associate with GLUT1 or GLUT2 in the absence of insulin signaling. Thus, our results show IR/GLUT1 or IR/GLUT2

complexes as recently reported for GLUT2 in rat hepatocytes (16). However, no involvement of GLUT2/IR association or of GLUT1/IR was made on basal hepatic glucose uptake in rat hepatocytes. Now, our data clearly suggest a direct role of IR in the molecular mechanism of hepatic glucose uptake independently of insulin action. Regarding selectivity of IR isoforms in the restoration of hepatic glucose uptake, both receptors have been related to differential insulin signaling in β-cells. Whereas isoform A is involved in insulin signaling leading to insulin gene transcription, isoform B signals through Akt leading to GK gene expression (21). In our hands, both IR_A and IR_B reconstituted hepatocytes signal through Akt. Thus, selectivity of IR isoforms in the restoration of hepatic glucose uptake because of differential signaling through Akt can be ruled out. Furthermore, our data on semiquantitative PCR show a very similar IR_A/IR_B ratio in neonatal hepatocytes. These results strongly suggest that isoform A predominates over isoform B of the IR in the regulation of glucose uptake by neonatal hepatocytes. Whether a shift in the IR_A/IR_B ratio may contribute to diminished IR-mediated glucose consumption by adult hepatocytes remains to be established.

Overexpression of IGF-IR increased glucose uptake in neonatal hepatocytes in the presence or absence of IR. Also, IGF-IR associated with GLUT1 or GLUT2 regardless of IR. These data also suggest a molecular mechanism of hepatic glucose uptake independently of insulin action. Taken together, our data suggest that either IR_A or IGF-IR works as GLUT1- or GLUT2-associated glucose cotransporter in neonatal hepatocytes. These results could shed some light on the diabetic phenotype regression that occurred at 4–6 months in LIRKO mice. At that time, LIRKO mice are normoglycemic and develop a liver dysfunction related to the occurrence of hyperplastic nodules. Thus, the lack of IR could be compensated by overexpression of IGF-IR, which in fact would enhance glucose uptake by the liver.

Exogenous expression of GLUT4 had no effect on basal glucose uptake in neonatal hepatocytes lacking or not IR, GLUT4 being mostly localized at the plasma membrane. Moreover, no association between GLUT4 and the IR was observed in GLUT4-expressing neonatal hepatocytes bearing or not IR. Although the GLUT family shows a high level of sequence homology and predicted secondary structure similarity, they show a differential targeting in polarized epithelial cells. Whereas GLUT1 and GLUT2 were predominantly targeted to the basolateral domain of the cells, insulin-regulatable GLUT4 was found in intracellular tubulovesicular structures beneath the surface of the cell (22). This differential localization might explain the lack of interaction of IR and GLUT4. However, hepatocytes expressing GLUT4 increased glucose uptake in response to insulin or IGF-I, this effect being independent of GLUT4 translocation into the plasma membrane. These data suggest a differentiation between GLUT4 translocation and its intrinsic activation in the insulin-induced glucose uptake, as previously suggested in muscle and adipose cells (23). In fact, mouse liver expresses most of the GLUT4-trafficking protein machinery, but not Vamp (vesicle-associated membrane protein) 2 (24, 25). More importantly, the effect of insulin on glucose uptake occurred without showing any association between GLUT4 and IR. Thus, IR works on glucose uptake through its specific association with endogenous, but not with exogenous, glucose transporters in neonatal hepatocytes.

In conclusion, type A, but not type B, IR plays a direct role in the regulation of glucose uptake/transport in neonatal hepatocytes. This effect is independent of insulin. Also, IGF-IR positively regulates glucose uptake by neonatal hepatocytes, regardless of IR. Thus, either type A IR or IGF-IR works on glucose uptake as a GLUT-1- or GLUT2-associated cotransporter. Exogenous expression of GLUT4 localizes into the plasma membrane, although GLUT4 does not increase basal glucose uptake in neonatal hepatocytes lacking or not IR. Hepatocytes expressing exogenous GLUT4 increase glucose uptake in the presence of insulin, without showing association between GLUT4 and IR. These results suggest that IR regulates glucose uptake as cotransporter through its specific association with endogenous, but not exogenous, glucose transporters in neonatal hepatocytes.

Acknowledgments

We give recognition to C. R. Kahn (Joslin Diabetes Center, Boston, MA) for kindly providing the pBabe hygro IR_A and pBabe hygro IR_B constructs and adeno Cre and A. M. Gómez-Foix (University of Barcelona, Barcelona, Spain) for the adeno GLUT4.

Received December 29, 2005. Accepted April 19, 2006.

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This work was supported by Grants SAF 2001/1302 and SAF 2002/0863, M.E.C. (Ministerio de Educacion y Ciencia) (Spain) and Red de Grupos de Diabetes Mellitus G03/212, and Instituto Carlos III, M.S.C. (Ministerio de Sanidad y Consumo) (Spain).

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Author Disclosure Summary: C.N., A.M.V., and M.B. have nothing to declare.

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